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2016-09

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Hermansson , M , Hanninen , S , Hokynar , K & Somerharju , P 2016 , ' The PNPLA-family phospholipases involved in glycerophospholipid homeostasis of HeLa cells ' , Biochimica and Biophysica Acta. Molecular and Cell Biology of Lipids , vol. 1861 , no. 9 , pp. 1058-1065 . <https://doi.org/10.1016/j.bbalip.2016.06.007>

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<http://hdl.handle.net/10138/224642>

<https://doi.org/10.1016/j.bbalip.2016.06.007>

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publishedVersion

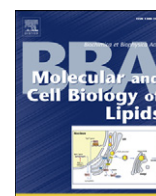
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# The PNPLA-family phospholipases involved in glycerophospholipid homeostasis of HeLa cells

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## ARTICLE INFO

### Article history:

Received 8 March 2016

Received in revised form 25 May 2016

Accepted 10 June 2016

Available online 15 June 2016

### Keywords:

Ca<sup>2+</sup>-independent phospholipase A2

Phospholipid

Degradation

Turnover

Lipid metabolism

## ABSTRACT

Mammalian cells maintain the glycerophospholipid (GPL) compositions of their membranes nearly constant. To achieve this, GPL synthesis and degradation must be coordinated. There is strong evidence that A-type phospholipases (PLAs) are key players in homeostatic degradation of GPLs, but the identities of the PLAs involved have not been established. However, some members of the Patatin-like phospholipase domain-containing proteins (PNPLAs) have been implicated. Accordingly, we knocked down all the PNPLAs significantly expressed in human HeLa cells using RNA interference and then determined whether the turnover of the major glycerophospholipids is affected by using mass spectrometry and metabolic labeling with stable isotope-labeled precursors. Knockdown of PNPLA9, PNPLA6 or PNPLA4 significantly (30–50%) reduced the turnover of phosphatidylcholine, –ethanolamine and –serine. In a notable contrast, turnover of phosphatidylinositol was not significantly affected by the knockdown of any PNPLA. Depletion of PNPLA9 and PNPLA4 also inhibited G<sub>0</sub>/G<sub>1</sub> to S cell cycle progression, which could thus be regulated by GPL turnover. These results strongly suggest that PNPLA9, -6 and -4 play a key role in GPL turnover and homeostasis in human cells. A hypothetical model suggesting how these enzymes could recognize the relative concentration of the different GPLs is proposed.

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## 1. Introduction

Glycerophospholipids (GPLs) form the lipid bilayer of all biological membranes and are also intimately involved in signal transduction, regulation of membrane trafficking and numerous other cellular phenomena [1]. Higher eukaryotes maintain the relative concentrations of the GPL classes within narrow limits [2,3], presumably because such homeostasis is vital for the organism. Consistently, hardly any human diseases exhibiting a significant deviation from the normal GPL composition exist, and deletion of a gene essential for the synthesis of a GPL is lethal in mice [4]. Yet, most GPLs are synthesized *via* more than one pathway (or enzyme isoform) supporting the notion that maintenance of GPL homeostasis is crucial. The key processes responsible for GPL

homeostasis are biosynthesis, remodeling (*i.e.*, exchange of a fatty acid residue) and degradation. Albeit the details of GPL homeostasis in mammals are still poorly understood [2], it is obvious that the biosynthesis and degradation of GPLs must be tightly coordinated [5]. Compelling evidence for this comes from studies where the synthesis of a GPL was boosted by over-expressing a rate-limiting synthetic enzyme in cultured cells. Thus the overexpression of *CTP:phosphocholine cytidyltransferase* boosted phosphatidylcholine (PC) synthesis several fold but, remarkably, the PC content of the cells was hardly increased [6–9]. Instead, the concentration of glycerophosphocholine (GPC), a deacylation product of PC, increased greatly. Analogously, boosting of the synthesis of phosphatidylethanolamine (PE) or phosphatidylserine (PS) did not significantly increase the cellular content of the respective GPL but its degradation increased in parallel [10,11]. Conversely, when the synthesis of a GPL was inhibited, its turnover decreased in proportion [12,13]. Obviously, mammalian cells must contain homeostatic phospholipases which “sense” the prevailing GPL composition and selectively degrade the GPL present in excess [2].

While the specific phospholipases involved in homeostatic degradation of GPLs have not been identified yet, certain PNPLA species have been implicated [2,8,14–17]. PNPLAs (a.k.a. Group VI Ca<sup>2+</sup>-independent phospholipases A or iPLA2s) comprise a family of mammalian lipid hydrolases with 9 members [18,19]. Some PNPLAs act as phospholipases hydrolyzing the *sn*1 or *sn*2 ester bond of an intact GPL or a lyso-GPL [17], while others mainly hydrolyze triacylglycerols *in vitro* [18,20]. Several

**Abbreviations:** GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPL, glycerophospholipid; LC, liquid chromatography; LPC, lysophosphatidylcholine; MS, mass spectrometry; PLA, A-type phospholipase; PNPLA, Patatin-like phospholipase domain-containing protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEP, phosphatidylethanolamine plasmalogen; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; RT-qPCR, reverse transcription quantitative PCR; siRNA, small interfering RNA; SM, sphingomyelin; TAG, triacylglycerol.

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PNPLAs also display a transacylase activity *in vitro* [17]. PNPLAs have been implicated in various biological processes including GPL acyl chain remodeling [14,21,22], release of polyunsaturated fatty acids from neuronal GPLs [23], removal of oxidized acyl chains from mitochondrial GPLs [24], stimulation of insulin secretion [25], cell growth [26], development of non-alcoholic fatty liver disease [27] as well as numerous other cellular processes and disorders (reviewed in [17,19,28]).

Regarding GPL homeostasis, overproduction of PC in Chinese hamster ovary cells greatly stimulated the production of PNPLA9 (a.k.a. iPLA2 $\beta$  or Group VIA Ca<sup>2+</sup>-independent PLA2) as well as GPC [9], thus implying that PNPLA9 could be responsible for the degradation of the excess PCs. However, studies where PNPLA9 was overexpressed in COS-7 cells did not support this possibility [29]. Also PNPLA6 (a.k.a. iPLA2 $\delta$  or NTE) has been implicated in GPL homeostasis [16], but this has not been firmly established.

Notably, several studies addressing the role of PNPLAs in GPL homeostasis have employed pharmacological inhibitors [2,17]. Such studies are, however, inconclusive since the inhibitors used are not selective for the targeted PLA [28]. In addition, in nearly all studies addressing the role of PLAs in GPL homeostasis the steady-state GPL composition of cells or tissues was determined. This approach can be problematic, since any (possible) effect on GPL turnover is likely to remain unnoticed due to the compensatory decrease of biosynthesis.

In this study, we first determined which PNPLAs are significantly expressed in HeLa cells by using RT-qPCR. Each PNPLA was then knocked down using RNA interference and the turnover of the major GPLs was determined by using deuterium-labeled GPL precursors and mass spectrometry. Notably, the knockdown of PNPLA4, PNPLA6 or PNPLA9 inhibited the turnover of PC, PE or PS markedly (by 30–50%). In contrast, the knockdown of the other PNPLAs had no detectable effect. Accordingly, these results provide strong evidence for that PNPLA4, PNPLA6 and PNPLA9 are important players in GPL homeostasis in mammalian cells.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Culture media and reagents were obtained from Invitrogen, D<sub>9</sub>-choline, D<sub>4</sub>-choline, D<sub>3</sub>-L-serine and D<sub>4</sub>-ethanolamine from Cambridge Isotope Laboratories (Andover, MA), D<sub>6</sub>-myo-inositol from CDN Isotopes (Quebec, Canada), <sup>35</sup>S-methionine from Perkin Elmer, phospholipase D (*Streptomyces* sp.), formic acid, ammonium formate and ammonium hydroxide from Sigma. All solvents (LC-MS grade) were from Fisher Scientific (Finland). Phospholipid standards were purchased from Avanti Polar Lipids (Alabaster, AL) or were synthesized as previously [30,31]. Their purities were confirmed by mass spectrometry and their concentrations were determined based on phosphate analysis [32].

### 2.2. Cell culture and RNA interference

HeLa cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin under 5% CO<sub>2</sub> at 37 °C. RNA interference was employed to knockdown the mRNAs of specific PNPLAs. The double stranded siRNA's used are listed in Supplemental Table S1. Each siRNA was used at the lowest effective concentration which varied from 2 to 10 nM. Cells grown to ~50% confluency on 35 or 60 mm dishes were transfected with a siRNA by using the Lipofectamine™ RNAiMAX reagent (Invitrogen) according to manufacturer's instructions up to 72 h.

### 2.3. RNA isolation and RT-qPCR

Total RNA was extracted from siRNA-treated cells by Machery-Nagel NucleoSpin® RNA II Kit (Biotop Oy, Finland) according to the

manufacturer's instructions. 2 µg of RNA was transcribed into cDNA using the RevertAid™ H Minus Reverse Transcriptase (Fermentas) and random hexameric primers. The qPCR reactions were carried out on Bio-Rad C1000™ Thermal Cycler in 20 µl using 5 µl of cDNA diluted by 1:6–1:20, 300 nM primers and the FastStart Universal SYBR Green Master-mix (Roche, Finland). The amplification (35–40 cycles) was carried out at 95 °C for 15 s, then at 55 °C for 30 s following 60 °C for 30 s. The data were analyzed by the CFX Manager™ 2.0 software. The qPCR primers (cf. Supplemental Table S2) were from Applied Biosystems or Oligomer (Helsinki, Finland).

### 2.4. Metabolic labeling

After 72 h of transfection the cells were washed with PBS and then incubated in DMEM containing 10% FBS, 1 mM hydroxylamine, D<sub>9</sub>-choline (100 µg/ml), D<sub>4</sub>-ethanolamine (100 µg/ml, adjusted to pH 7.0 by acetic acid), D<sub>3</sub>-L-serine (300 µg/ml) and D<sub>6</sub>-myo-inositol (100 µg/ml). In the pulse-chase experiments the cells were incubated in the labeling medium for 4 or 24 h, washed thrice with DMEM and then incubated in DMEM containing 10% FBS, 1 mM hydroxylamine, unlabeled choline (500 µg/ml), ethanolamine (500 µg/ml), L-serine (1000 µg/ml) and myo-inositol (500 µg/ml) up to 24 h. After washing thrice with PBS, the cells were scraped into 2 ml of ice-cold water, the suspension was vortexed and aliquots were withdrawn for protein concentration determination. The remaining suspension was mixed with 3 ml of ice-cold methanol and stored at –20 °C. To assess the effect of RNAi on the rate of protein synthesis, the cells were transfected with a siRNA as above (cf. Section 2.2) and then [<sup>35</sup>S]-methionine (10 µCi/ml) was added to the medium. After incubation for up to 3 h, the cells were washed thrice with PBS, scraped into 2 ml of PBS, pelleted by centrifugation and suspended in RIPA lysis buffer (Thermo Scientific). Aliquots were withdrawn for protein concentration determination and the remaining sample was mixed with Optisafe scintillation fluid to determine its radioactivity by scintillation counting.

### 2.5. Lipid analysis

The lipids were extracted [33] with added internal standards and after evaporation of the solvent the lipids were dissolved in methanol/chloroform (2:1, v/v) and stored at –20 °C before the analysis with LC-MS. The lipids were separated on a Waters Acquity UPLC system equipped with an Acquity BEH-C<sub>18</sub> 1.0 × 150 mm column using a solvent gradient described previously [34]. The eluent was infused to the ESI source of a Waters Quattro Micro triple-quadrupole mass spectrometer operated in the positive ion mode and the individual lipid species were detected using selective reaction monitoring (SRM). The precursor ions were [M + H]<sup>+</sup> for PC, SM, PE, PS and [M + NH<sub>4</sub>]<sup>+</sup> for PI. The product ion was phosphocholine for PC or SM and diacylglycerol for PE, PS and PI. Triacylglycerols (TAG) were detected as [M + NH<sub>4</sub>]<sup>+</sup>-ions. The concentrations of the individual lipid species were determined from the SRM or ion chromatograms using the QuanLynx and TargetLynx software tools (Waters). To assess GPL turnover rates the labeled-to-unlabeled-ratio for each individual GPL-class was plotted against chase time and the label half-time was calculated from fits of an exponential decay model to the data points using OriginPro 8.5 (OriginLab, MA).

### 2.6. Analysis of water-soluble lipid metabolites

To determine the labeling of the water-soluble choline, serine or ethanolamine metabolites the cells were incubated with deuterium-labeled choline, serine and ethanolamine as above (cf. Section 2.4). They were then washed with PBS and subjected to lipid extraction [33] after addition of the D<sub>4</sub>-choline, propanolamine and threonine standards. The upper phase was recovered, evaporated to dryness under a nitrogen stream and the residue was reconstituted in

methanol/water (1:1, v/v). The choline metabolites were analyzed directly by LC-MS [35], while the ethanolamine and serine metabolites were first converted to dansylated derivatives [36] which were then quantified with LC-MS using SRM [34].

### 2.7. Cell cycle analysis

The cells were transfected with a siRNA for 72 h as above (cf. Section 2.2), collected and the level of PNPLA knockdown was determined with RT-qPCR. The cells were fixed with ethanol and the DNA was stained using the FxCycle™ PI/RNase staining solution (Life Technologies) according to the manufacturer's protocol. The cells were then analyzed on a BD Accuri™ C6 flow cytometer and the data were processed with the BD Accuri™ C6 1.0 software. Cell doublets were eliminated using the side scatter pulse height versus side scatter pulse area dot plot. Expression of the proliferation marker Ki-67 (gene *MKI67*) was determined using RT-qPCR [37].

### 2.8. Other assays

Published methods were used to quantify protein [38] and total lipid phosphorus [32].

## 3. Results

### 3.1. Knockdown of the PNPLAs expressed in HeLa cells

The mammalian PNPLA family consists of 9 members *i.e.*, PNPLA1 - PNPLA9 [18]. By using RT-qPCR we found that in HeLa cells PNPLAs 2, 3, 4, 6, 8 and 9 are expressed at a significant level while PNPLAs 1, 5 and -7 were not detectable (Supplemental Fig. S1). Accordingly, we targeted each of the 6 significantly expressed PNPLAs with a specific siRNA for 72 h and then quantified the respective mRNAs with RT-qPCR. The targeted mRNA was reduced by 70–95% depending on the PNPLA (Supplemental Table S3, Fig. S2). The PNPLA9 and PNPLA6 proteins were also markedly reduced in the respective cells as determined by Western blotting (Supplemental Figs. S3 & S4). The levels of the other PNPLA proteins could not be determined since suitable antibodies were not available. We also measured how depletion of a PNPLA affected the expression of non-targeted PNPLAs (Supplemental Fig. S2). Modest fluctuations in the levels of the non-targeted PNPLA mRNAs were observed and thus possible compensatory effects cannot be completely excluded.

### 3.2. Effect of PNPLA depletion on the cellular lipid composition

HeLa cells were treated with a control siRNA or siRNA targeting a PNPLA for 72 h (cf. Section 2.2 Materials and methods) and the protein, lipid phosphate, TAG and cholesterol contents of the cells were measured. A modest (~15%) increase in the lipid phosphate was observed in the cells depleted of PNPLA2, -6 or -9, while no change was detected

in those depleted of PNPLA4, -3 or -8 (Table 1). The phospholipid class (Table 1) or the phospholipid molecular species (data not shown) compositions of the cells were only slightly affected by the knockdown of each PNPLA. However, the knockdown of PNPLA6 or -9 diminished the concentration of phosphatidic acid (PA) by ~50% while the knockdown of PNPLA2, -3 or -4 had a lesser effect on this lipid. Depletion of PNPLA6 also caused ~2.5-fold increase in the lyso-PC content of the cells. In the cells depleted of PNPLA2, -4 or -9 TAG increased by approx. 3-, 3- or 2-fold, respectively. In summary, the depletion of each PNPLA had only a minor effect on the membrane lipid composition of HeLa cells, but the TAG content of the cells depleted of PNPLA2, -4 or -9 increased markedly.

### 3.3. Effect of PNPLA depletion on phospholipid turnover

Although the depletion of none of the PNPLAs significantly altered the GPL compositions of HeLa cells, the turnover of GPLs could have been affected because the synthesis and degradation are coordinated (cf. Section 1 Introduction). To test this, HeLa cells were pretreated with a specific siRNA for 72 h and then incubated with deuterium-labeled choline, ethanolamine, serine and inositol for 24 h, washed and chased in a medium containing the respective unlabeled precursors. Hydroxylamine (1 mM), an inhibitor of PS decarboxylase, was added to the media to prevent decarboxylation of PS to PE, thus avoiding overlapping MS signals from the serine- and ethanolamine-labeled PE species. After lipid extraction, GPLs were quantified by LC-MS and the labeled/unlabeled ratio was determined for the major GPL classes and sphingomyelin vs. the chase time (Fig. 1). First, in the cells depleted of PNPLA9 the label was chased from PC much more slowly (half-time 17.5 h) than from the control cells (half-time 8.5 h) (Fig. 1; Table 2). Second, the turnover of the diacyl and plasmalogen PE classes was also markedly reduced (Fig. 1; Table 2). The labeling of these GPLs still increased during the early chase, most probably because the pool of cellular (phospho)ethanolamine is relatively large and thus only slowly diluted by cold (phospho)ethanolamine. Third, the turnover of PS was nearly abolished when PNPLA9 was knocked down (Fig. 1). Fourth, in a sharp contrast to the other GPLs, the turnover of PI was not significantly influenced (Fig. 1). The turnover rate of SM could not be measured since its labeling continued to increase until the end of the chase, most probably due to that SM receives its head group from PC.

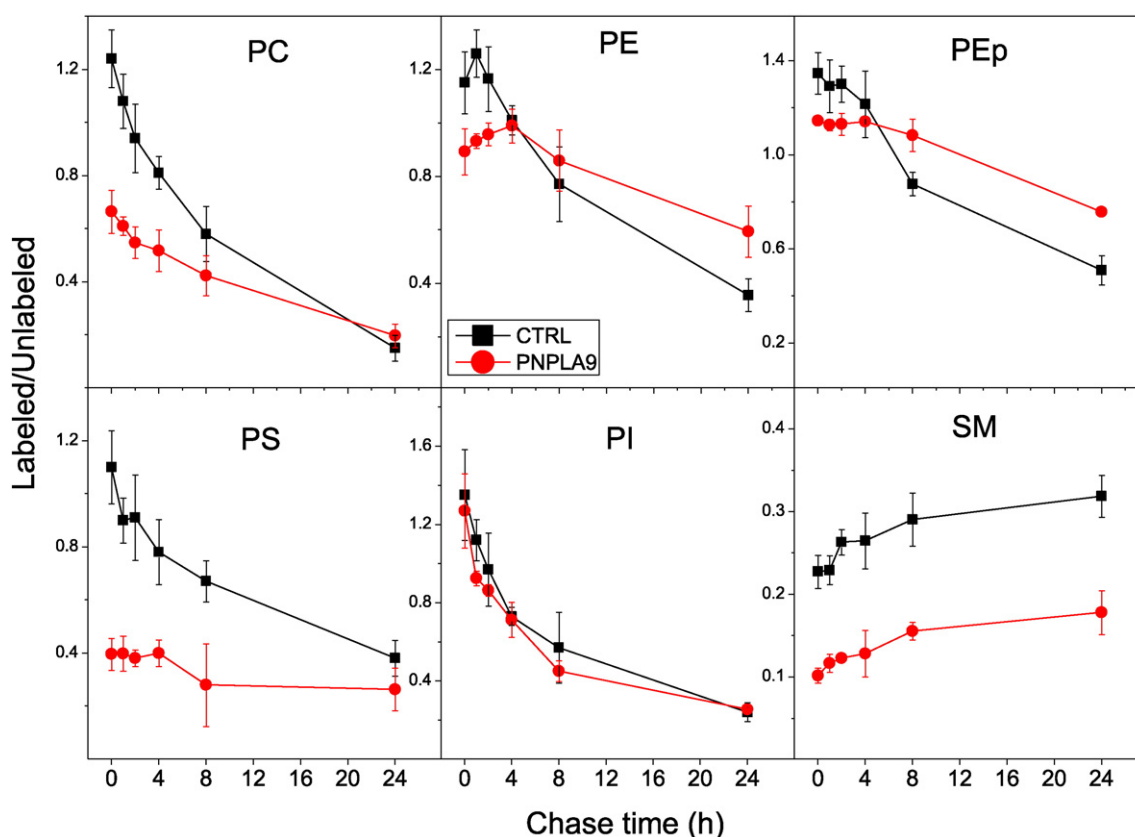
The turnover data obtained after the knockdown of the other PNPLAs is summarized in Table 2. The knockdown of PNPLA6 reduced the turnover of PC, PE and PS similarly to PNPLA9, while in the PNPLA4-depleted cells the turnover of these GPLs was somewhat less affected. In contrast to those PNPLAs, depletion of PNPLA8 had only a small effect on the turnover of PC, PE and PS, and no effect was detected upon the depletion PNPLA2 or PNPLA3. Turnover of PI was not affected in any of the knockdown cells (Table 2). Comparable results were obtained when a shorter time of labeling (4 h) was employed (data not shown).

**Table 1**

Lipid class compositions of PNPLA-depleted HeLa cells. HeLa cells were treated with a control siRNA or siRNA targeting the indicated PNPLA for 72 h, washed and then incubated in the growth medium for 24 h. The cellular lipid and protein contents were then quantified as described under Materials and Methods (Sections 2.5 & 2.8). Data are mean of 3 independent experiments  $\pm$  SD.

|        | nmol/mg protein  |                | mol%           |                |               |               |               |               |               |               |
|--------|------------------|----------------|----------------|----------------|---------------|---------------|---------------|---------------|---------------|---------------|
|        | Lipid phosphorus | TAG            | PC             | PE             | PEp           | PS            | PI            | PA            | LPC           | SM            |
| CTRL   | 135 $\pm$ 7      | 3.7 $\pm$ 0.3  | 55.5 $\pm$ 3.1 | 16.5 $\pm$ 0.3 | 4.3 $\pm$ 0.3 | 6.2 $\pm$ 1.2 | 4.7 $\pm$ 0.6 | 4.3 $\pm$ 0.4 | 1.5 $\pm$ 0.2 | 6.8 $\pm$ 0.3 |
| PNPLA2 | 164 $\pm$ 16     | 12.1 $\pm$ 1.6 | 54.6 $\pm$ 0.7 | 17.4 $\pm$ 1.5 | 4.9 $\pm$ 0.4 | 6.6 $\pm$ 0.9 | 4.8 $\pm$ 0.5 | 2.4 $\pm$ 0.7 | 1.5 $\pm$ 0.1 | 7.7 $\pm$ 0.4 |
| PNPLA3 | 145 $\pm$ 3      | 3.6 $\pm$ 0.6  | 55.1 $\pm$ 2.8 | 14.9 $\pm$ 2.7 | 3.9 $\pm$ 0.3 | 8.0 $\pm$ 0.2 | 4.8 $\pm$ 1.4 | 2.9 $\pm$ 0.3 | 1.8 $\pm$ 0.5 | 8.3 $\pm$ 0.8 |
| PNPLA4 | 148 $\pm$ 3      | 11.5 $\pm$ 2.7 | 55.2 $\pm$ 2.0 | 15.8 $\pm$ 0.8 | 4.1 $\pm$ 0.4 | 8.3 $\pm$ 0.5 | 5.4 $\pm$ 1.3 | 3.0 $\pm$ 0.5 | 1.4 $\pm$ 0.2 | 6.4 $\pm$ 0.5 |
| PNPLA6 | 156 $\pm$ 5      | 4.5 $\pm$ 1.3  | 54.4 $\pm$ 0.1 | 16.4 $\pm$ 0.9 | 3.8 $\pm$ 0.8 | 6.3 $\pm$ 0.4 | 4.8 $\pm$ 0.3 | 1.8 $\pm$ 0.1 | 3.6 $\pm$ 0.2 | 8.6 $\pm$ 1.4 |
| PNPLA8 | 138 $\pm$ 8      | 3.8 $\pm$ 0.3  | 56.1 $\pm$ 2.0 | 15.2 $\pm$ 2.6 | 4.3 $\pm$ 0.6 | 6.5 $\pm$ 0.9 | 4.6 $\pm$ 0.4 | 3.8 $\pm$ 0.2 | 1.7 $\pm$ 0.1 | 7.6 $\pm$ 0.4 |
| PNPLA9 | 153 $\pm$ 8      | 8.0 $\pm$ 2.0  | 54.1 $\pm$ 3.3 | 20.3 $\pm$ 1.5 | 4.0 $\pm$ 0.3 | 7.4 $\pm$ 0.8 | 5.0 $\pm$ 1.2 | 1.6 $\pm$ 0.3 | 1.4 $\pm$ 0.1 | 6.0 $\pm$ 0.5 |





**Fig. 1.** Effect of PNPLA9 depletion on GPL head group turnover. HeLa cells were treated with a non-silencing siRNA (black squares) or a siRNA targeting PNPLA9 (red circles) for 72 h and were then incubated in a medium containing D<sub>3</sub>-choline, D<sub>4</sub>-ethanolamine, D<sub>3</sub>-L-serine, D<sub>6</sub>-myo-inositol and hydroxylamine for 24 h. The cells were then washed and chased in a medium containing the respective unlabeled precursors. The labeled and unlabeled lipids were quantified by LC-MS as detailed under [Materials and methods](#). The data are mean of 3 independent experiments  $\pm$  SD.

### 3.4. Effect of PNPLA knockdown on the complete deacylation of PC and PE

Complete deacylation of PC and PE leads to formation of glycerophosphocholine (GPC) or -ethanolamine (GPE), respectively. Since previous reports indicated that pharmacological (nonspecific) inhibition of PNPLAs reduces the levels of these metabolites [8,39], we assessed whether the depletion of PNPLA9, -6 or -4 would reduce the concentrations and/or labeling of GPC and GPE. HeLa cells were transfected with a siRNA for 72 h, incubated with deuterated choline and ethanolamine for 24 h and the labeled and unlabeled GPC and GPE were determined by LC-MS. The total GPC content of the control, PNPLA9, -6 or -4 siRNA-treated cells were  $23.2 \pm 2.1$ ,  $16.8 \pm 0.8$ ,  $16.2 \pm 3.3$  and  $15.1 \pm 2.2$  nmol/mg protein, respectively. Thus depletion of PNPLA9, -6 or -4 reduced the concentration of GPC by 30–35%. Consistently, the labeling of GPC in the PNPLA9 or PNPLA6-depleted cells was

reduced by 20–25% (Supplemental Fig. S5). Also the GPE content of the knockdown cells was markedly reduced. *i.e.*, the GPE content of the control cells and PNPLA9, -6 or -4 siRNA-treated cells were  $0.90 \pm 0.11$ ,  $0.45 \pm 0.13$ ,  $0.63 \pm 0.05$  and  $0.36 \pm 0.21$  nmol/mg protein, respectively. The glycerophosphoserine content of the cells was too low for meaningful analyses. In summary, these results are consistent with involvement of PNPLA4, -6 and -9 in the deacylation of PC and PE.

### 3.5. Effect of PNPLA depletion on GPL synthesis

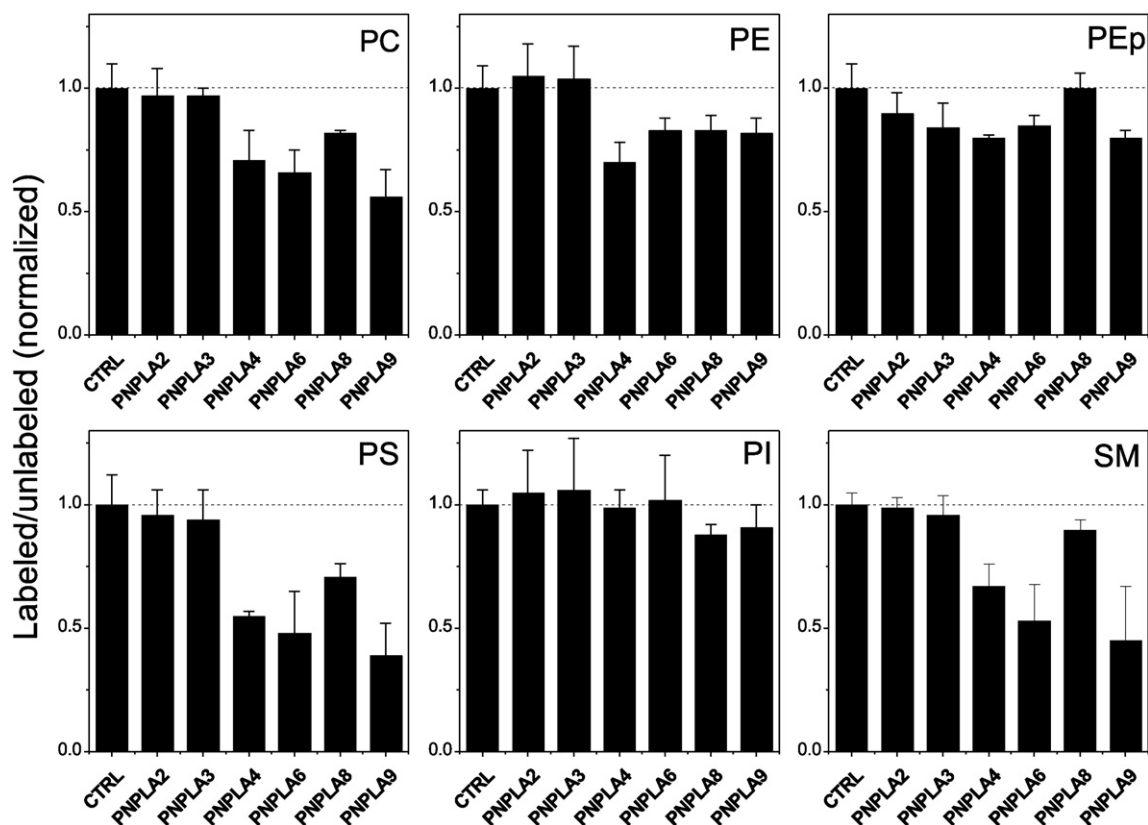
As shown in Fig. 1., the labeling of PC, PE, PEp, PS or SM was significantly reduced in the cells depleted of PNPLA9 after a 24 h pulse, indicating that the synthesis of those GPLs was markedly reduced in the knockdown cells. Therefore, the labeling of those lipids in the knockdown cells was investigated (Fig. 2). Besides PNPLA9, the depletion of PNPLA4, -6 or -8 significantly reduced the labeling of the GPLs, while the knockdown of PNPLA2 and -3 had no effect. As expected, the labeling of PI was not affected by the knockdown of any PNPLA. Since the cells do not accumulate the phospholipids significantly (Table 1), this result supports the notion that, beside turnover, the synthesis of PC, PE and PS is inhibited in the cells depleted of PNPLA4, -6, or -9. The inhibition of synthesis is most likely indirect, *i.e.*, it is due to a homeostatic response to the reduced GPL degradation in the respective knockdown cells.

To further assess the effect of the depletion of PNPLA4, -6 or -9 on the metabolism of PC we determined the specific labeling of the water-soluble precursors of PC and its deacylation product GPC (Fig. S5). The labeling of choline, phosphocholine and CDP-choline or GPC was only modestly or insignificantly affected by the depletion of any of these PNPLAs. The knockdown of PNPLA4, -6 or -9 slightly reduced the labeling of phosphocholine and CDP-choline, consistently with the

**Table 2**

Effect of PNPLA9 depletion on GPL head group turnover. HeLa cells were treated with a control siRNA or a siRNA targeting the indicated PNPLA for 72 h and then the turnover of the different GPLs were determined as detailed under [Materials and methods](#). Data are mean of 3–5 independent experiments  $\pm$  SD.

|        | Label half-time (h) |                |                |                 |               |
|--------|---------------------|----------------|----------------|-----------------|---------------|
|        | PC                  | PE             | PEp            | PS              | PI            |
| CTRL   | $8.5 \pm 0.9$       | $13.6 \pm 2.1$ | $16.3 \pm 2.8$ | $18.4 \pm 2.2$  | $6.8 \pm 0.8$ |
| PNPLA2 | $9.2 \pm 1.1$       | $14.3 \pm 1.4$ | $16.6 \pm 2.4$ | $19.2 \pm 2.4$  | $5.9 \pm 1.1$ |
| PNPLA3 | $9.0 \pm 1.4$       | $13.1 \pm 2.0$ | $15.7 \pm 1.8$ | $20.1 \pm 2.2$  | $6.1 \pm 1.3$ |
| PNPLA4 | $15.7 \pm 1.4$      | $17.8 \pm 2.6$ | $25.3 \pm 3.7$ | $43.2 \pm 3.2$  | $6.4 \pm 1.2$ |
| PNPLA6 | $17.4 \pm 4.2$      | $24.1 \pm 4.4$ | $27.7 \pm 3.2$ | $44.5 \pm 4.5$  | $7.9 \pm 1.1$ |
| PNPLA8 | $10.2 \pm 1.1$      | $17.5 \pm 2.7$ | $18.3 \pm 1.9$ | $33.4 \pm 3.3$  | $6.6 \pm 0.5$ |
| PNPLA9 | $17.6 \pm 2.9$      | $19.8 \pm 3.2$ | $29.8 \pm 4.4$ | $54.2 \pm 13.4$ | $8.8 \pm 1.3$ |



**Fig. 2.** Effect of PNPLA depletion on synthesis of phospholipids. HeLa cells were treated with a control siRNA or siRNA targeting a PNPLA for 72 h and were then incubated with D<sub>3</sub>-choline, D<sub>4</sub>-ethanolamine, D<sub>3</sub>-L-serine and D<sub>6</sub>-myo-inositol for 24 h. The labeled and unlabeled lipids were quantified by LC-MS as described under [Materials and methods](#) and the labeled-to-unlabeled ratios were plotted relative to that in the control cells. Data are mean of 3–5 independent experiments  $\pm$  SD.

reduced labeling of PC (*cf.* Fig. 2). No significant differences were observed in the specific labeling of the PE precursors ethanolamine, phosphoethanolamine and CDP-ethanolamine or the PS precursor serine (data not shown).

### 3.6. Effect of PNPLA depletion on cell growth

We consistently observed that the depletion of PNPLA9 resulted in a markedly reduced number of cells per dish (up to 50% at 72 h of transfection), while the depletion of PNPLA2, -3, -4, -6 or -8 had a somewhat smaller effect on the cell number (~20% reduction). To assess whether the lower cell number was due to a perturbed cell cycle, as suggested previously for PNPLA9 [40], the cells were stained with propidium iodide and analyzed by flow cytometry. In the cells depleted of PNPLA9 or PNPLA4 (but not in those depleted of other PNPLAs) the cell cycle was apparently perturbed as indicated by a ~30% decrease in the fraction of cells in S/G<sub>2</sub>/M-phases (Supplemental Fig. S6). Consistently, the expression of the proliferation marker MKI67 [37] was reduced by ~30% upon depletion of PNPLA4 and PNPLA9 (Supplemental Table S4). These data suggest that a turnover of GPLs mediated by PNPLA9 or PNPLA4 is required for a normal G<sub>1</sub> to S cell cycle progression. Intriguingly, however, cell cycle was not impaired in the PNPLA6-depleted cells despite that the turnover of GPLs was markedly inhibited in those cells as well.

## 4. Discussion

### 4.1. PNPLAs are key mediators of GPL turnover in HeLa cells

Previous studies have shown that the turnover of PC is mainly mediated by PLAs in many cell lines, including HeLa [8,15,41,42]. However, the particular PLAs involved in this process have remained

obscure. Here we show that the depletion of PNPLA4, -6 and -9 markedly (30–50%) inhibited the turnover of PC as well as PE and PS in HeLa cells. Consistent with the reduced turnover of PC and PE in the knock-down cells, the amounts of GPC and GPE, the respective deacylation products, were reduced by 30% in the cells that were depleted of PNPLA9, -6 or -4. Complete cessation of the turnover of those GPLs was not achieved, which could be due to incompleteness of PNPLA depletion (Table S3), compensatory increases in non-targeted PNPLAs (Fig. S2) or other phospholipases not studied here, or due to that no single PNPLA is responsible for the degradation a GPL. To the latter option, we attempted knocking down two PNPLAs simultaneously but, unfortunately, such double knockdown was either inefficient or toxic to the cells for unknown reasons. The fact that PNPLAs 4, 6, and 9, when knocked down individually, had a clear effect on turnover PC PE and PS suggests that collectively they probably account for most of the extralysosomal turnover of those lipids. Whether these PNPLAs act individually or in concert is not clear based on the current data, but apparently their relative contributions vary depending on the GPL class. For instance, PNPLA6 and -9 together may fully account for the turnover of PS, while PNPLA4 seems to have only a minor role. On the other hand, PNPLA4, -6 and -9 seem to contribute similarly to the turnover of PC, PE and PEp. Presently it is not clear to whether the PNPLAs indicated in GPL turnover act as a PLA1, PLA2 or lysophospholipase in HeLa cells. It is possible that they act either on different substrates within a class or on different residues on the same substrates (*e.g.* sn1 or sn2 chain) and the products are subsequently hydrolyzed by another PNPLA. The fact that lysophospholipase activity of PNPLA6 is far higher than its phospholipase activity *in vitro* [43] implies that this protein may act mainly as a lysophospholipase in HeLa cells as well. Consistently, we found that lyso-PC accumulated 2.5-fold in the cells depleted of PNPLA6 (Table 1).

We emphasize that the present data by no means exclude the involvement of other phospholipases in GPL homeostasis. It has been

previously suggested that phospholipase C (PLC) plays a role in the turnover of PC and PE in HeLa cells [44]. In the present study, the turnover of PI was not influenced by the knockdown of any PNPLA, which suggests that PI turnover could be mediated by a PLC. Also the lysosomal phospholipases most probably contribute to the degradation of cellular GPLs, but their contribution is not clear at present.

Although abundantly expressed in HeLa cells (Fig. S1), the knockdown of PNPLA2 or -3 had no significant effect on GPL turnover. This is not unexpected since these enzymes have very low PLA-activity *in vitro* and hydrolyze mainly TAGs [20]. PNPLA2 (a.k.a. ATGL) is probably the main TAG hydrolase in mammals [45]. Consistently, we find that the knockdown of PNPLA2 in HeLa cells led to a 3-fold increase in their TAG content (Table 1). In contrast, we did not observe an increase in TAG content upon depletion of PNPLA3, consistently with that TAG does not accumulate in the PNPLA3 knockout mouse [46].

PNPLA4 efficiently hydrolyzes TAGs and retinyl esters, but has a very low PLA activity *in vitro* [20,47,48]. Thus it is intriguing that in the present study the depletion of PNPLA4 had a major impact on GPL turnover. This may indicate that the protein requires some cofactors, e.g. other proteins, lacking the *in vitro* studies, for an efficient hydrolysis of GPLs *in vivo*. On the other hand, depletion of PNPLA4 markedly increased the TAG content of HeLa cells (Table 1) consistently with the TAG hydrolase activity ascribed to this protein [20]. Whether the reduced GPL turnover and TAG accumulation are linked is unclear, since TAG did not accumulate in the cells depleted of PNPLA6 (Table 1).

Global knockout of PNPLA6 is embryonic-lethal in mouse [49] but mice with a conditional knockout in the brain are viable [50]. These mice exhibit elevated PC in the frontal cortex [51], which indicates the role of PNPLA6 in PC homeostasis in this tissue. In HeLa cells, the knockdown of PNPLA6 was shown to halve cellular GPC-content without affecting steady-state PC-levels [52], consistently with the present study.

In neuronal cells of a PNPLA8 knockout mouse, cardiolipin was significantly elevated, PEp was depleted and the molecular species profiles of several GPLs were altered [53], thus showing that PNPLA8 plays a crucial role in GPL metabolism in neuronal cells of mouse. In the present study, the metabolism of the major GPLs (cardiolipin was not studied) was not perturbed upon the knockdown of PNPLA8. These contrasting results could indicate that PNPLA8 participates in GPL homeostasis in mouse but not in humans, or alternatively, its function may vary between cell types.

Consistent with the present data, early work on PNPLA9 suggested that this enzyme could play a “housekeeping” role in mediating basal PC turnover and generating lysophospholipid acceptors for incorporation of polyunsaturated fatty acids (e.g. arachidonate) to GPLs [14,21,22,54], albeit not in all cell types [55]. On the other hand, PNPLA9 knockout mouse has neuronal problems [17,19], yet the GPL compositions of its tissues are unaltered [56,57]. While this could be taken to indicate that PNPLA9 is not involved in GPL homeostasis in mouse, it is possible that this is not the case if coordination of synthesis and degradation maintains the GPL composition constant. Besides greatly reducing GPL turnover, depletion of PNPLA9 led to accumulation of TAG in the present study (Table 1). It is not clear whether this is due to that PNPLA9 acts also as a TAG hydrolase or because inhibition of PC and PE synthesis (secondary to their diminished degradation) increases the concentration of diacylglycerol and, consequently, that of TAG. Intriguingly, expression of PNPLA2 doubled in cells depleted of PNPLA9 (Fig. S2), which suggests that depletion of PNPLA9 could lead to a loss of TAG hydrolase activity which would (partially) be compensated by elevated PNPLA2. Clearly, establishing whether this is the case requires further experiments.

#### 4.2. Effect on cell cycle

The cells depleted of PNPLA9 or PNPLA4 contained approx. 30% less cells in S/G2/M phases (Supplemental Fig. S3) and the cell number per plate was markedly reduced. Consistently, depletion of PNPLA9 has

been shown to inhibit the G<sub>1</sub> to S transition, and to reduce the number of cells per dish [40,58,59]. By which mechanism the depletion of the PNPLAs affects the cell cycle or growth is not clear. However, as PNPLA4 has been shown to facilitate transacylation of retinol and accumulation of retinyl esters [48], the inhibition of the cell cycle could be connected to retinol signaling that has been shown to inhibit HeLa cell proliferation [60]. Interestingly, consistent with a previous report [52], cell cycle appeared to proceed normally in the PNPLA6-depleted cells despite a marked reduction in GPL turnover. This indicates that the cell cycle progression is not directly coupled to GPL turnover. Possibly, PNPLA9 and PNPLA4, but not PNPLA6, produces lipid mediator(s) required for the cell cycle progression. Further investigations are required to resolve the mechanism by which PNPLA9 and PNPLA4 promote cell proliferation.

#### 4.3. Coordination of GPL synthesis and degradation

Besides degradation, knockdown of PNPLA4, -6 and -9 inhibited the synthesis of the major GPLs as indicated by a reduced labeling from a head group precursor. Consistently, the level of phosphatidic acid, a precursor of all GPLs, was diminished in the knockdown cells (Table 1). Yet, the inhibition of labeling of the different GPLs correlated with the inhibition of their turnover (cf. Figs. 1 and 2), i.e., the synthesis decreased in the order of PS > PC > PE > PEp while that of PI was unaffected. Consistent with diminished PC synthesis, labeling of phosphocholine or CDP-choline was also reduced in those knockdown cells. Together, these data strongly support the previous results indicating that the synthesis and degradation of GPLs are tightly coordinated in mammalian cells. Such coordination would explain why the steady-state GPL composition of HeLa cells did not change significantly when PNPLA4, -6 or -9 was depleted (Table 1).

While it is obvious that GPL synthesis and degradation must be tightly coordinated in mammalian cells to maintain GPL homeostasis, it is unclear how such coordination is achieved. It is possible that specific regulator proteins sensing the GPL composition of cellular membranes exist, and that such proteins regulate the homeostatic phospholipases and synthetic enzymes. However, there is little if any experimental evidence for such sensory proteins. It is also difficult to understand how such proteins, if existing, would accurately “sense” the relative concentrations of the many different GPLs, as required to maintain homeostasis, and how would they relay this information to the enzymes carrying out the synthesis and degradation so that these opposing processes are accurately coordinated. Regarding this, we have proposed a simple mechanism which could in principle accomplish such integration [2,30]. The mechanism is based on the so-called superlattice (or regular distribution) model which suggest that different lipids *tend* to adopt regular, rather than random lateral distribution in membranes [61,62]. From this putative principle it necessarily follows that a limited number of “critical” or optimal compositions with particular stoichiometries exist. Another important prediction of the model is that when the composition deviates from a critical one, the molecules present “in excess” would have an elevated chemical activity and, consequently, an increased propensity to efflux from a membrane, which would make them susceptible for hydrolysis by homeostatic PLAs. When the molecules present in excess have been degraded, hydrolysis would stop or slow down dramatically thus preventing excessive hydrolysis. Notably, we have recently provided strong evidence that PNPLA9 is regulated by substrate efflux propensity [63] and thus, potentially, by the tendency of the membrane lipids to adopt regular distributions. Importantly, such a tendency could also regulate the biosynthesis since the GPL molecules present “in excess” should have increased chemical activity and could thus potentially inhibit the synthetic enzymes e.g. by a feedback mechanism (cf. [61,62]). Accordingly, highly accurate coordination of synthesis and degradation could be achieved by this simple mechanism based on composition-dependent changes in the chemical activity of different



GPLs. However, further experimentation is required to assess the validity of such putative homeostatic mechanisms.

In conclusion, the present data provide compelling evidence that three PNPLA proteins play a key role in GPL degradation in HeLa cells. It remains to be seen if those proteins play this role in other human cells and tissues as well.

## Transparency document

The Transparency document associated with this article can be found, in the online version.

## Acknowledgements

We wish to thank Ms. Tarja Grundström for expert technical assistance. This work was supported by grants from the Finnish Academy, Sigrid Jusélius Foundation, Jenny and Antti Wihuri Foundation, Biomedicum Helsinki Foundation and Magnus Ehrnrooth Foundation. MH was a Fellow of the Sigrid Jusélius Foundation. The funding sources had no role in study design, the collection, analysis and interpretation of data, writing this report or in the decision to submit it for publication.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbailp.2016.06.007>.

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